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Cutting Edge Communication

Engraftment of Post 5-Fluorouracil Murine Marrow into Minimally Myeloablated (100 cGy) Murine Hosts

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ABSTRACT

Minimal myeloablative approaches are now being widely applied in the treatment of different hematological malignancies. One hundred cGy whole-body irradiation is a stem-cell-toxic, relatively nonmyelotoxic treatment that allows for relatively high levels of donor chimerism. 5-Fluorouracil (5-FU) treatment leads to a relative concentration of high proliferative potential–colony-forming cell (HPP-CFC) and is an approach that has been used to induce in vivo progenitor/stem cell cycling to facilitate retroviral integration in gene therapy approaches. We have now evaluated the capacity of marrow harvested 1, 2, 6, or 12 days after 5-FU treatment (150 mg/kg) to engraft in 100 cGy-treated female BALB/c mice. Engraftment was assessed at 3, 10, and 24 weeks. A rapid induction of an engraftment defect occurred 1 day post 5-FU and persisted through day 6 with a recovery by day 12. To evaluate cell cycle status of normal and 5-FU-treated marrow cells, male donors received hydroxyurea (900 mg/kg i.v.) or phosphate-buffered saline (PBS), 2 h prior to marrow harvest and transplantation into submyeloablated female recipients. Engraftment levels were similar for hydroxyurea-treated mice and controls. Thus, these studies show transiently defective engraftment of 5-FU-treated marrow into submyeloablated hosts, which may be related to the cell cycle status of the stem cells.

INTRODUCTION

THE DOGMA THAT MYELOABLATION is required to open space for marrow stem cell engraftment has been thoroughly challenged (1–4). We have shown that moderate bone marrow engraftment levels could be obtained when high cell doses were injected into untreated hosts (5,6), and that higher levels of engraftment are seen with lower levels of marrow cells when normal marrow is infused into 100 cGy-treated mice (7). In addition, a high level of engraftment persisted for at least 12 months after transplant, and toxicity was negligible. This latter approach is a stem cell toxic, nonmyelotoxic treatment, and the high levels of engraftment seem to depend upon altering the host-to-donor stem cell ratios. Marrow harvested after 5-fluorouracil (5-FU) injection is generally enriched in primitive cycling HPP-CFC progenitors and short-term repopulating cells, but when tested in nontreated hosts was found to be markedly defective in engraftment (8). Post 5-FU marrow has been of particular

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interest as a source of cycling marrow cells that might be suitable for retroviral gene therapy approaches. The capacity of post-5-FU marrow to engraft in the minimally treated host has not previously been assessed. Here we evaluate marrow, spleen, and thymus engraftment, in 100 cGy-treated mice, of marrow cells harvested 1, 2, 6, and 12 days after 150 mg/kg 5-FU. We have evaluated donor chimerism at 3, 10, and 24 weeks after infusion.

MATERIALS AND METHODS

Mice

BALB/c H-2^d mice were purchased from Charles River Laboratories (Wilmington, MA) and housed in a conventional clean facility for at least 1 week before experimental use. All mice received mouse chow and acidified water ad libitum.

Marrow cell suspension

Mice were sacrificed by cervical dislocation. Bone marrow was flushed from femurs and tibias with cold phosphate-buffered saline (PBS). The cells were counted in a hemocytometer, washed, and resuspended for injection in PBS.

Recipients conditioning

BALB/c females received a submyeloablative radiation dose of 100 cGy. Radiation was given at a rate of 92–94 cGy/min in one fraction using a cesium-137 gamma source (Gamma cell 40; Nordian, Kanata, Ontario, Canada) 2–4 h prior to transplant.

Transplantation from 5-FU-treated donors

Male donors received 5-FU (150 mg/kg i.v.) (Hoffman-La Roche) or PBS (GIBCO-BRL, Bethesda, MD) 1, 2, 6 or 12 days prior to bone marrow collection. The equivalent of one femur and one tibia was resuspended into 0.5 ml of PBS. Female recipients were transplanted by tail vein injections for 2 consecutive days with donor marrow or with PBS. Recipient mice (average 6) were sacrificed 3 weeks, 10 weeks, or 6 months post-transplant. The percentage of male cells in bone marrow, spleen and thymus was analyzed by Southern blotting and by fluorescent in situ hybridization (FISH).

Transplantation from hydroxyurea-treated donors

To assess the stem cell cycle relationship with engraftment, we used hydroxyurea, which is known to kill cells in S phase (9–11). Hydroxyurea half-life is less than 1 h, allowing elimination of cells cycling at the time of transplantation (12). In addition to 5-FU treatment, male donors received either hydroxyurea (900 mg/kg i.v.) (Sigma Chemical Co., St. Louis, MO) or PBS 2 h prior to bone marrow harvest. Transplant and analysis were performed in the same way as described for 5-FU-treated donors.

Southern blot analysis

DNA extracts of bone marrow were prepared by lysis in 150 mM NaCl, 20 mM Tris (pH 7.5), 20 mM EDTA, 1% sodium dodecyl sulfate (SDS), 0.5 mg/ml proteinase K, and 50 μg/ml pancreatic RNase A at 55°C overnight. Purification was completed by organic extraction with phenol-chloroform and ethanol precipitation. Five micrograms of each DNA sample were digested with *Dra* I (Boehringer Mannheim, Mannheim, Germany), and separated by gel electrophoresis in 0.8% agarose (GIBCO-BRL, Bethesda, MD). DNA fragments were alkaline-transferred onto Zetaprobe nylon membranes (Biorad, Richmond, CA). The presence of Y chromosome-specific DNA sequences was assessed using a pY2-cDNA probe (13,14) (donated by Dr I. Lemischka, Princeton University). Sample loading variability was assessed and adjusted for by reprobing membranes with a cDNA for interleukin-3 (IL-3) (donated by J. Ihle and DNAX, Palo Alto, CA). Probes were labeled with ^{32}P using a random-primed labeling kit (Boehringer Mannheim), and autoradiography was carried out using Kodak XRP X-ray film (Eastern Kodak, Rochester, NY). Blots were exposed to phosphorimaging plates (Molecular Dynamics, Sunnyvale, CA) and scanned with a 400A phosphorimager (Molecular Dynamics). Boxes were plotted around the bands and the raw intensity of IL-3 and pY2 signals was used for calculation. To correct for loading variability, pY2 signal was divided by the IL-3 signal. For background correction, the average signal of female controls (2 per blot) was subtracted. The average male signal (2 per blot) was used to determine the 100% value. Percent engraftment was calculated as follows: % engraftment = (sample pY2/IL3-female pY2/IL3)/(male pY2/IL3-female pY2/IL3) × 100.

FISH

Cytospins were fixed in 50% Carnoy's (75% methanol/25% glacial acetic acid) and 50% PBS solution for 10 min before being baked at 72°C for 1 h. The slides were then further fixed in 100% Carnoy's for 5 min before permeabilization using enzymatic digestion with 0.2 μg/ml proteinase K (Sigma Diagnostics, St. Louis, MO) in 20 mM Tris buffer at 37°C for 1.5 min. The cytospins were dehydrated through graded ethanol, then denatured in 70% formamide (GIBCO-BRL) in 2× SSC (0.3 M

NaCl and 30 mM sodium citrate, pH 6.4) at 70°C for 3 min. The slides were dehydrated once more, and hybridized with a digoxigenin-labeled Y chromosome probe at 45°C overnight. Unbound probe was removed by stringent washing in three changes of 50% formamide in 2× SSC and two changes of 2× SSC at 45°C. Following a wash in 4× SSC (0.6 M NaCl and 60 mM sodium citrate, pH 6.4) at room temperature, the slides were blocked using a blocking buffer consisting of 5% fetal calf serum (FCS), 5% nonfat milk (Shaw's, Bridgewater, MA), and 0.05% Triton X-100 (Sigma) in 4× SSC for 5 min. Detection of digoxigenin was done using antidigoxigenin-rhodamine, Fab fragments (Boehringer Mannheim), at a concentration of 6.5 μg/ml in PBS containing 2% bovine serum albumin fraction V (Sigma) for 30 min in the dark. Unbound antibody was removed through three extensive, light-protected, washings: first in 4× SSC for 10 min, then in 4× SSC containing 0.05% Triton X-100 for 10 min, and finally in 4× SSC for 10 min. Cytopsins were counterstained in 0.4 μmol 4,6-diamidino-2-phenylindole (DAPI) (Sigma) and mounted with Vectashield (Vector, Burlingame, CA). Specific positive label was confirmed under the microscope by a visual check at excitation and emission wavelengths other than that of rhodamine. At least 200 cells from three different fields were analyzed per sample.

Statistical analysis

For transplant analysis, the percentage of engraftment was calculated taking a male control as 100% and a female control as 0%. The nonparametric Wilcoxon rank-sum test is used for comparison. The level of statistical significance was set at 0.05 for comparison of treatment groups with controls of the same day (e.g., day 1, 5-FU vs. PBS). For pair-wise intergroup comparison (e.g., 5-FU day 1 to day 12 comparison), the level of statistical significance was adjusted to the number of multiple comparisons and set at $0.05/6 \approx 0.01$.

Correlation between levels of engraftment in bone marrow, spleen and thymus was assessed using the nonparametric Spearman rank test. Engraftment data is presented as mean ± standard error of the mean. All p values are two sided.

RESULTS

Engraftment of 5-FU-treated donor marrow

Engraftment observed 3 weeks after transplant determined by Southern blot is presented in Table 1. The level of engraftment in bone marrow, spleen, and thymus of day 1 post 5-FU was extremely low and significantly decreased compared to day 6 post 5-FU and control marrow. At 10 weeks and 6 months post transplant, the level of engraftment increased in all organs, confirming that low-dose irradiation allows high levels of long-term engraftment, even of 5-FU treated marrow (Fig. 1). A significant engraftment defect was observed as early as 1 day after 5-FU administration and persisted until day 6 after 5-FU (all $p < 0.05$). A complete recovery of engraftment was observed 12 days after 5-FU treatment as compared to PBS-treated donors (Fig. 1). Examples of FISH performed on cytospun bone marrow cells and Southern blot analysis of spleen engraftment are shown in Figs. 2 and 3.

TABLE 1. ENGRAFTMENT 3 WEEKS AFTER TRANSPLANT

	Bone marrow	*Spleen*	*Thymus*
D1 post 5-FU	1 ± 1	1 ± 0	0
D1 post PBS	24 ± 8	33 ± 3	8 ± 1
D6 post 5-FU	33 ± 11	17 ± 6	0

Mean level of engraftment (%) ± standard error is indicated. The equivalent of two tibias and two femurs from male mice were transplanted into sublethally irradiated mice (100 cGy). Levels of engraftment were determined by percentage of male cells assessed by Southern blot using a Y-specific probe. There were 3 mice per group.

At 10 weeks and 6 months post transplant, bone marrow engraftment was significantly higher in the day 12 post 5-FU group as compared to days 1, 2, and 6 (all $p < 0.01$). The increase was also observed for spleen and thymus, but the statistical significance was not obtained (all $p < 0.02$).

Cell cycle status of engrafting marrow

To evaluate cell cycle status of normal and 5-FU-treated marrow cells, male donors received hydroxyurea (900 mg/kg) i.v. or PBS 2 h prior to marrow harvest. We first analyzed the effect of hydroxyurea on engraftment of untreated marrow. Our data showed that hydroxyurea-treated marrow displayed similar engraftment levels compared to PBS treated marrow (Table 2). We then studied the effect of hydroxyurea on marrow previously treated with 5-FU. Cells were injected into submyeloablated females. Engraftment levels analyzed at 10 weeks and 6 months post-transplant in all three organs showed no statistical difference for hydroxyurea-treated mice compared to those receiving PBS (Figs. 4 and 5). The pattern of recovery of post 5-FU marrow at 10 weeks post infusion differed between the experiments presented in Figs. 1 and 4. This presumptively represents intrinsic biologic variability.

Combining FISH results for bone marrow at 10 weeks and 6 months, we calculated that the proportion of engrafting cells that were quiescent was 89.6 ± 1.8% of 5-FU-treated mar-

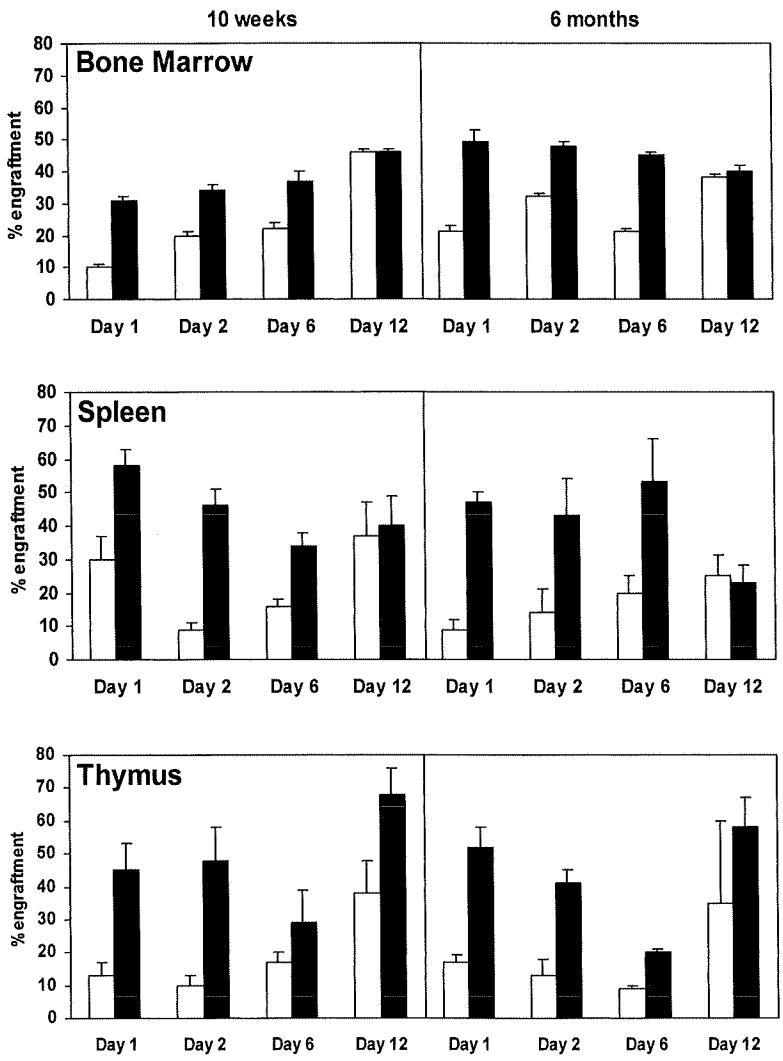

FIG. 1. Effect of 5-FU administration on engraftment in bone marrow, spleen, and thymus. Donor males were injected with either 5-FU (150 mg/kg) (□) or PBS (■) 1, 2, 6, or 12 days before bone marrow transplantation into submyeloablated (100 cGy) females. Percentage of male engraftment was assessed at 10 weeks and 6 months post transplant using a Y chromosome-specific probe by FISH for bone marrow, and by Southern blot for spleen and thymus.

row ($n = 54$) and 80.0 ± 3.9% of control marrow ($n = 23$, $p = 0.0198$). This confirmed that engrafting cells in 5-FU-treated marrow are mostly noncycling.

Correlation of engraftment data within organs

A significant correlation was found between engraftment in bone marrow and spleen ($r = 0.37$, $p < 0.01$), bone marrow and thymus ($r = 0.42$, $p < 0.01$), and spleen and thymus ($r = 0.28$, $p < 0.01$).

DISCUSSION

In this study, we have demonstrated a clear defect of engraftment after donor exposure to 5-FU. The model chosen used 100-cGy recipient conditioning to assess donor marrow repopulating capacity. Low-dose irradiation has proven to be an effective technique to obtain high levels of engraftment in a BALB/c male into female transplant model (7). Whereas day 1, 2, and 6 post-5FU exposure showed a marked defect of engraftment (at 10 and 24 weeks post-transplant), a complete recovery was seen by day 12 post 5-FU treatment. The pattern of recovery was slightly different for 3 weeks engraftment where the nadir was shorter with recovery by day 6. The different results at 3 weeks as contrasted to 10 and 24 weeks are consistent with different kinetics of engraftment of cytokine treated marrow; here, at early time points (up to 3 weeks) post cell infusion, engraftment was normal, whereas a progressively more profound engraftment defect was observed at longer times post cell infusion (15).

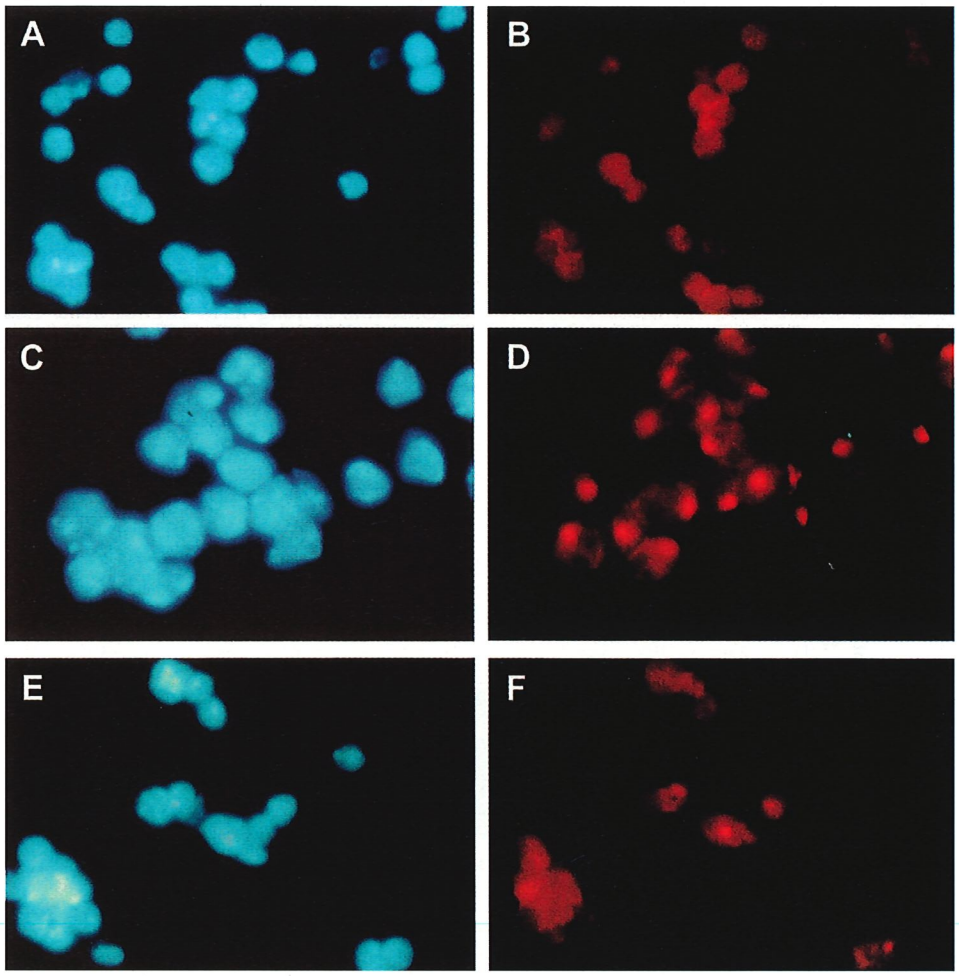

FIG. 2. Evaluation of male engraftment by FISH. Bone marrow cytospins from BALB/c mice: female negative control (**A** and **B**), male positive control (**C** and **D**) and female 10 weeks post transplant of day 12 post 5-FU male marrow (**E** and **F**). Marrow cells were stained using in situ hybridization for a digoxigenin-conjugated Y chromosome-specific "painting" probe and rhodamine anti-digoxigenin and visualized using a 590-nm longpass filter (*right*). DAPI was excited with UV and visualized using a 450-nm longpass filter (*left*). Positive cells are of donor origin whereas unlabeled cells are of host origin. Slides were mounted in Vectashield antifade. Original magnification, 313×.

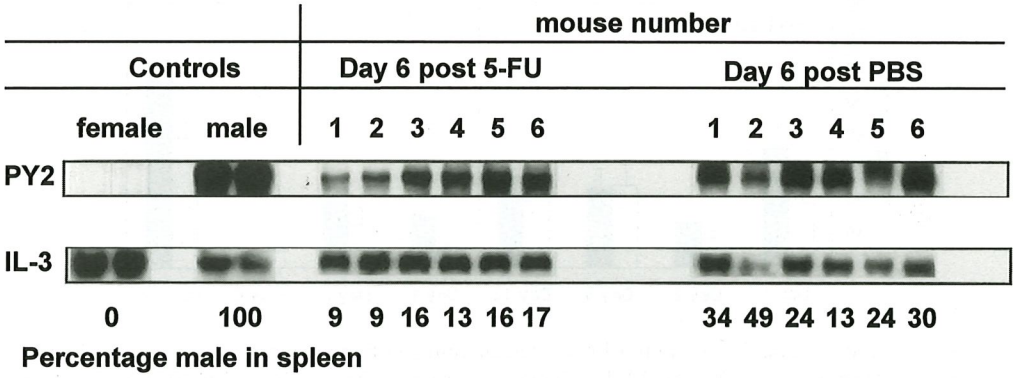

FIG. 3. DNA analysis of female mice transplanted with day 6 post 5-FU male marrow. DNA was extracted 10 weeks after transplant and digested with *Dra*I. Southern blots were probed with pY-2. The IL-3 gene was used to correct the DNA loading. Autoradiographs were exposed for 3 days with intensifying screens.

TABLE 2. EFFECT OF HYDROXYUREA ON ENGRAFTMENT AT 10 WEEKS AND 6 MONTHS POST TRANSPLANT OF NORMAL BONE MARROW

	10 Weeks			*6 Months*		
	BM	*SP*	*THY*	*BM*	*SP*	*THY*
HU	30 ± 3	37 ± 3	52 ± 7	32 ± 2	24 ± 3	ND
PBS	32 ± 2	25 ± 3	57 ± 5	46 ± 2	28 ± 4	ND

Mean level of engraftment (%) ± standard error is indicated. Hydroxyurea (900 mg/kg) or PBS was injected 2 h before marrow harvest. Levels of engraftment were determined using a Y-specific probe by FISH for bone marrow and Southern blot for spleen and thymus. There were 6–9 mice per group. ND; Analysis not done. Autoradiographs were exposed for 3 days with intensifying screens.

These data are consistent with different classes of stem cells being responsible for early and late engraftment. Alternatively, 5-FU treatment or cytokine exposure could convert long-term populating cells into short-term populating cells. They are also consistent with data indicating that cycling stem cells have an engraftment defect (16–18).

Our data confirmed the previous observation (7) that submyeloablation is an effective technique for obtaining high levels of long-term engraftment. These high levels of engraftment were obtained without specific toxicity. No animal died of immediate or delayed toxicity. Two animals died of unknown cause 5 months post-transplant in PBS-treated groups. Marrow from mice treated with 5-FU, despite being defective in engraftment, can also

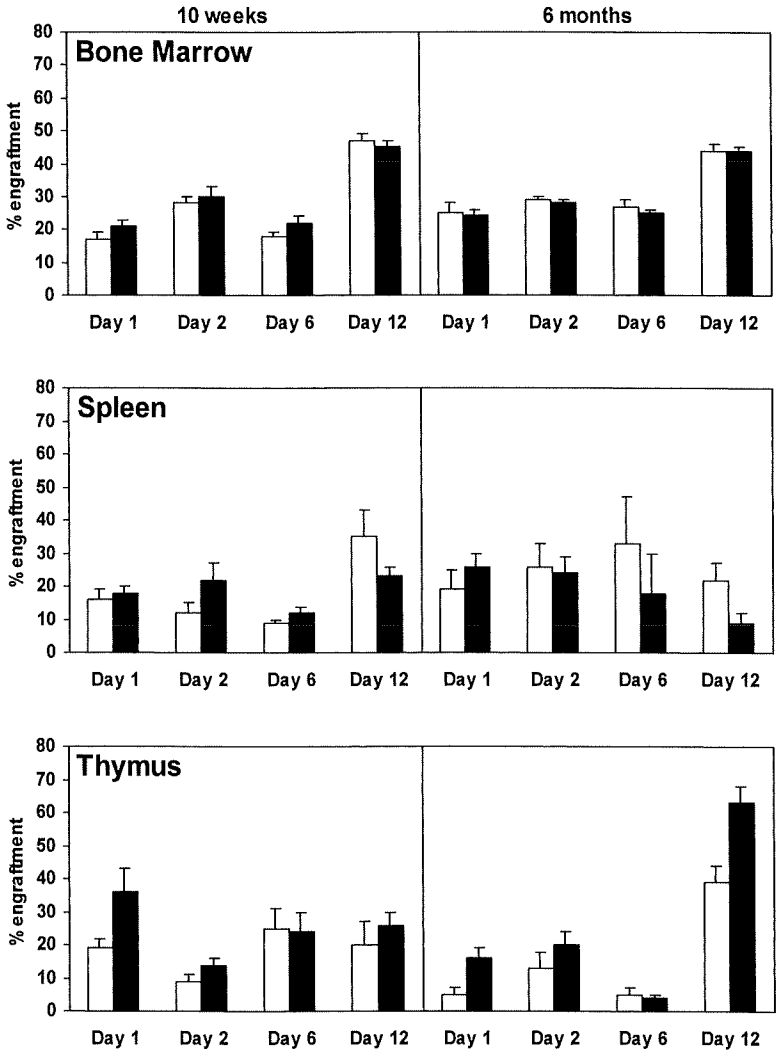

FIG. 4. Effect of hydroxyurea administration on 5-FU treated engraftment in bone marrow, spleen, and thymus. Donor males were injected with 5-FU (150 mg/kg) 1, 2, 6, or 12 days before harvest and either hydroxyurea (900 mg/kg) (□) or PBS (■) 2 h before collection. Bone marrow cells were injected into submyeloablated (100 cGy) females. Percentage engraftment was assessed at 10 weeks and 6 months post transplant by FISH using a Y chromosome-specific painting probe for bone marrow, and by Southern blot for spleen and thymus. Most groups consisted of 6 mice each (range n = 3–10).

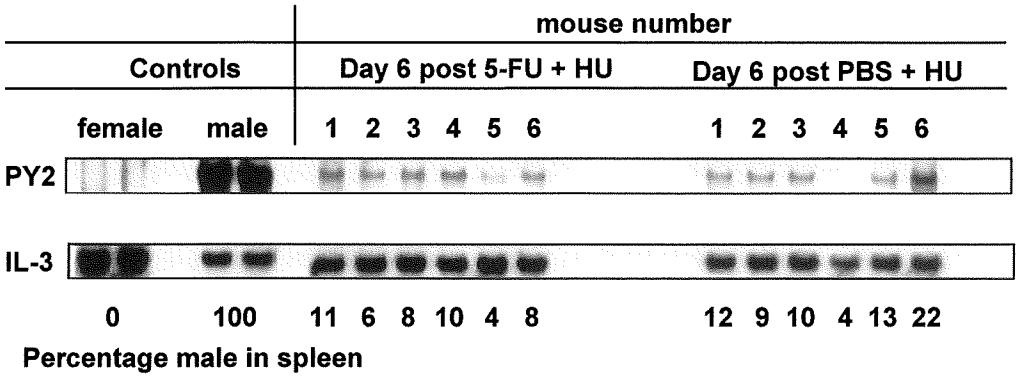

FIG. 5. Southern blot analysis of spleen engraftment 10 weeks post transplant. Day 6 post 5-FU male marrow was transplanted in submyeloablated (100cGy) female recipient 2 h after hydroxyurea (900 mg/kg) or PBS injection. Extracted DNA was digested with *Dra*I, blotted, and then hybridized with a Y chromosome-specific probe. The IL-3 gene was used to correct loading.

achieve high levels of long-term engraftment. High levels of engraftment were observed in marrow, spleen, and thymus and correlate with each other. Thus, we can infer that high chimerism was obtained in both myeloid and lymphoid lineages, knowing that bone marrow cellularity is predominantly myeloid and that spleen is mainly lymphocytic. The previously observed 5-FU-induced engraftment defect in nonmyeloablated recipients (8) is consistent with the present results. The kinetics of this deficit appeared comparable in submyeloablated hosts: rapid induction 1 day post 5-FU, persistence through day 6, and recovery by day 12. In contrast, the nadir seemed displaced from day 6 (nonablated hosts) to day 1 (100 cGy-treated hosts).

Hydroxyurea suicide studies indicated that engrafting cells both from normal or post 5-FU mice were noncycling. Hydroxyurea specifically kills cells in mitotic S phase. We showed no major effect of hydroxyurea on engraftment when given to donors 2 h before marrow harvest. The proportion of noncycling cells that engrafted was significantly higher in the 5-FU-treated group compared to controls, but this small difference is probably not biologically significant. These data confirm the observation by Ramshaw et al. (6) that engrafting cells from normal or 5-FU-treated marrow are predominantly noncycling. This phenomenon probably explains in part the deficit of engraftment induced by 5-FU, as it is known to spare primitive hematopoietic stem cells but induces them to enter cell cycle (19–21).

Previous work has indicated that 100 cGy conditioning is nonmyelotoxic, but stem cell toxic (7). We have also established that 100cGy treatment of donors reduced cell engraftment capacity to 8.6% of normal controls (22). The hypothesis that engraftment is determined by the donor-to-host cell ratio is supported by the high levels of engraftment observed in our study. In this case, the final host-to-donor ratio is determined by competition between hosts and donor stem cells. In BALB/c mice, the total cellularity of bone marrow was measured as 525 ± 34 million determined by whole-skeleton crushing in separate animals (23). If 100 cGy reduces the total host-repopulating activity to 8.6% of 525 million, 45 million host cells will remain. Using 40 million donor cells, we should expect about 47% engraftment, which approximates the numbers observed in the animals given control marrow. Electron microscopy studies of bone marrow after irradiation have shown endothelial injuries at doses as low as 100 cGy, but tissues seemed to heal rapidly. At higher doses, injuries increased and appeared long lasting (24). These lesions can have a deleterious impact on marrow homing. Hendrix et al. showed that homing was reduced in irradiated marrow compared to controls (25).

These data confirm that submyeloablation is a useful approach to obtain high levels of sustained marrow, spleen, and thymus engraftment, even if the donor bone marrow has been exposed to chemotherapy. In addition, clinical applications in genetic diseases (e.g., thalassemia or sickle cell anemia) or cancer treatment will need to consider the temporal kinetics of engraftment when marrow is obtained from 5-FU-treated donors.

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